The hitchhiker's guide to the voltage-gated sodium channel galaxy

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Eukaryotic voltage-gated sodium (Na_v) channels contribute to the rising phase of action potentials and served as an early muse for biophysicists laying the foundation for our current understanding of electrical signaling. Given their central role in electrical excitability, it is not surprising that (a) inherited mutations in genes encoding for Na_v channels and their accessory subunits have been linked to excitability disorders in brain, muscle, and heart; and (b) Na_v channels are targeted by various drugs and naturally occurring toxins. Although the overall architecture and behavior of these channels are likely to be similar to the more well-studied voltage-gated potassium channels, eukaryotic Na_v channels lack structural and functional symmetry, a notable difference that has implications for gating and selectivity. Activation of voltage-sensing modules of the first three domains in Na_v channels is sufficient to open the channel pore, whereas movement of the domain IV voltage sensor is correlated with inactivation. Also, structure–function studies of eukaryotic Na_v channels show that a set of amino acids in the selectivity filter, referred to as DEKA locus, is essential for Na^+ selectivity. Structures of prokaryotic Na_v channels have also shed new light on mechanisms of drug block. These structures exhibit lateral fenestrations that are large enough to allow drugs or lipophilic molecules to gain access into the inner vestibule, suggesting that this might be the passage for drug entry into a closed channel. In this Review, we will synthesize our current understanding of Na_v channel gating mechanisms, ion selectivity and permeation, and modulation by therapeutics and toxins in light of the new structures of the prokaryotic Na_v channels that, for the time being, serve as structural models of their eukaryotic counterparts.

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Introduction

Animals use electrical signals to encode and propagate vital information, often over long distances (Hille, 2001). To this end, a diverse family of membrane protein complexes known as ion channels contains hydrophilic pathways across cell membranes that catalyze the otherwise energetically unfavorable flow of charged ions through the lipid bilayer. Consequently, ion channels generate and take advantage of a transmembrane voltage gradient that constitutes a key element in cellular communication. In mammals, voltage-gated sodium (Na_v) channels play an important role in fast electrical signaling because they have a Na^+-selective transmembrane pathway that can open and close rapidly (i.e., gate) in response to changes in membrane voltage, thereby regulating the Na^+ permeability of the cell membrane and generating the rapid upstroke of action potentials (Hodgkin and Huxley, 1952b; Catterall, 2012; Fig. 1 A). As such, Na_v channels are widely targeted by clinical therapeutics as well as toxins from numerous venomous animals and plants (Kaczorowski et al., 2008; Kalia et al., 2015). Abnormal Na_v channel activity stemming from inherited or spontaneous mutations in Na_v channel genes can also lead to various diseases, termed channelopathies, which can manifest as both hypo- and hyper-excitable phenotypes (Wood et al., 2004; George, 2005; Cannon, 2006; Dib-Hajj and Waxman, 2010; Jurkat-Rott et al., 2010; Mantegazza et al., 2010). In the former, such mutations can result in deficient expression and loss of Na^+ current, whereas in the latter, defective channel inactivation can produce excessive Na^+ entry that results in prolonged or unstable depolarization. For example, >1,000 mutations in neuronal Na_v channels are associated with a spectrum of epilepsy syndromes (Claes et al., 2009). Moreover, alterations in the functional properties of Na_v channel isoforms that are preferentially expressed in the skeletal muscle or in the heart muscle are associated with neuromuscular diseases and cardiac pathologies, respectively (George, 2005;

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Abbreviations used in this paper: BTX, batrachotoxin; HH, Hodgkin and Huxley; K_v, voltage-gated potassium; Na_v, voltage-gated sodium; PM, pore module; STX, saxitoxin; TTX, tetrodotoxin; VSD, voltage-sensing domain; VTD, veratridine.

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Cannon, 2006). In some cases, Na_v channel abnormalities can cause excruciating pain sensations, or in rare instances, isoform-specific loss of function phenotypes can eliminate the sensation of pain altogether (Dib-Hajj et al., 2013; Leipold et al., 2013).

In humans, nine Na_v channel pore–forming α subunits have been identified ($Na_v1.1$–$Na_v1.9$; Fig. 1 B), with amino acid homology predicting a similar domain and transmembrane architecture: the pore-forming α subunit consists of four connected parts (domains (D)I–IV), each having six transmembrane segments (S1–S6; Catterall, 2000). These homologous domains are similarly configured and consist of a voltage-sensing domain (VSD; S1–S4), which contains positively charged residues along the S4 helix, and a portion of the structure that forms the sodium ion–selective pore (S5–S6) that can partially open after each of the DI–III voltage sensors has moved in response to changes in membrane voltage (Fig. 1, C–F).

Typically, heterologous expression of the Na_v channel α subunit by itself is sufficient for generating Na^+ currents in most eukaryotic cell expression systems. In vivo, however, Na_v channels act as a multi-protein membrane-embedded signaling complex (Abriel and Kass, 2005), chief among these being auxiliary β subunits (β1–4) that modify the expression and gating properties of the pore domain as well as contribute to cell migration and adhesion (O'Malley and Isom, 2015). Their importance in proper Na_v channel function is reflected in mutations that result in neurological and cardiac syndromes (Namadurai et al., 2015). Recently reported crystal structures of β3 and β4 have uncovered intricate interactions of these elements within the Na_v channel signaling complex (Gilchrist et al., 2013; Zhang et al., 2013a; Namadurai et al., 2014). Moreover, these and other studies established new roles for β subunits in influencing Na_v channel pharmacology and as potential therapeutic targets (Gajewiak et al., 2014). Consistent

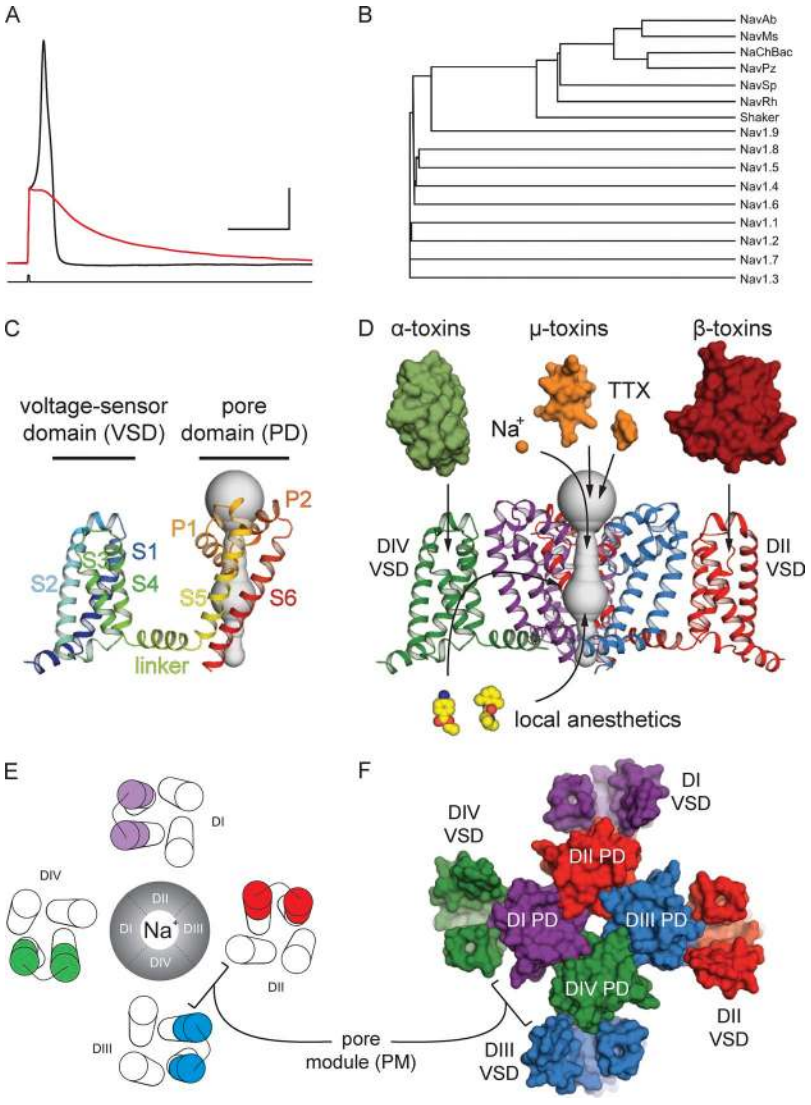

Figure 1. Na_v channel function, family tree, and structural architecture. (A) Evoked action potential recorded from a mouse DRG neuron at room temperature before (black) and after (red) the application of 1 μM TTX. X axis is 30 ms, and y axis is 20 mV. (B) A phylogenetic tree of Na_v channels as well as Shaker obtained using Vector NTI AlignX software. (C) The side view of a signal subunit of the Na_vAb channel homotetramer (Protein Data Bank accession no. 3RVY) in ribbon style is colored from N terminus (blue) to C terminus (red). This view highlights the VSD as a modular four-helix bundle. (D) Side view of the Na_vAb channel with the front VSD and pore domain removed for clarity. For illustrative purposes, Na_vAb is colored according to a pseudotetrameric arrangement expected for eukaryotic Na_v cannels. Representative classes of protein toxins (α, β, and μ), small molecule toxins (TTX), as well select small molecule drugs (lidocaine and benzocaine) are represented with arrows pointing to their presumed canonical binding sites on the channel. (E) Top-view schematic of a eukaryotic Na_v channel with the S3b–S4 region of the VSDs from different domains is highlighted in different colors. The ion-conducting Na^+ pore is found in the center of this view. (F) A structural top view of the Na_vAb channel colored according to a pseudotetrameric arrangement expected for a eukaryotic Na_v channel (as in D). This subunit coloring highlights the "domain-swapped arrangement" of the VSDs around the PM observed for all voltage-gated ion channels.

with their role as central cell-signaling hubs in excitable cells, Na_v channels interact with a myriad of cellular constituents including but not limited to calmodulin (Kink et al., 1990), contactin, fibroblast growth factor homologous factors, ankyrin, clathrin-interacting protein 1A, mitogen-activated protein kinase, and neural precursor cell-expressed developmentally down-regulated protein 4 (Dib-Hajj and Waxman, 2010).

Structural insights into eukaryotic Na_v channel function lag compared with the structural revolution that is leading the understanding of voltage-gated potassium (K_v) channels (Long et al., 2005a). Recently, the discovery of biochemically more tractable bacterial Na_v (or $BacNa_v$) channels set the stage for several experimental structure determinations of six-transmembrane homotetrameric channels (Na_vAb, Na_vRh, and Na_vCt) and two-transmembrane pore module (PM)-only structures (Na_vMs and Na_vAe; Payandeh et al., 2011, 2012; McCusker et al., 2012; Zhang et al., 2012b; Tsai et al., 2013; Shaya et al., 2014). These simpler $BacNa_v$ channels collectively highlight the basic design principles of the more complex eukaryotic Na_v channels in unprecedented detail (Payandeh and Minor, 2015). However, these significant advances are only tempered by the still unknown structural and functional correlations to eukaryotic Na_v channels. For one, the homotetrameric $BacNa_v$ channels will show inherent mechanistic differences in the cooperativity of their gating, as well as their interactions with permeant ions and therapeutics when compared with pseudo-heterotetrameric eukaryotic Na_v channels. Moreover, the inherent lack of symmetry in the mammalian Na_v channel protein sequence raises basic questions about the role of individual domains in their functional properties. Even so, the $BacNa_v$ channels may be suitable models for understanding the mechanisms that underlie the biology of pseudo-symmetric eukaryotic Na_v channels. For example, all full-length $BacNa_v$ channel structures revealed a central ion PM with a domain-swapped arrangement in which each individual VSD is offset by one step from its pore domain, around the perimeter of the fourfold structure (Fig. 1 D; Payandeh et al., 2011, 2012; Zhang et al., 2012b; Tsai et al., 2013). This architecture likely underlies an important aspect of the electromechanical coupling mechanism (Long et al., 2005b) and was foreshadowed by receptor site–mapping studies in eukaryotic Na_v channels that suggested certain toxins contact the VSD in one homologous domain and the PM of another (Cohen et al., 2007; Leipold et al., 2007).

In light of the recent advances in structural biology, we anticipate that experimental structures of eukaryotic Na_v channels will become available in the near future. This would undoubtedly provide new insights into some of the long-standing questions in the ion channel field. Inspired by this prospect, we will broadly survey the current state of our understanding of the mechanisms that underlie Na_v channel function as well as their modulation by ligands in this Review. Prokaryotic Na_v channel structures and their implications on our understanding of the eukaryotic sodium channels will also be discussed. We hope that this Review adequately captures the storied history of Na_v channels and will also catalyze new studies of these fascinating molecules.

Gating mechanisms

Voltage gating. According to the Hodgkin and Huxley (HH) model, changes in membrane permeability during an action potential are controlled by redistribution of voltage-dependent gating particles between two permissive positions (Hodgkin and Huxley, 1952a,c,d). The sodium–ion conductance is determined by the activating "m" and inactivating "h" particles. Na_v channels open when all three "m" particles move into the up state, whereas activation of the slower moving "h" particle produces the phenomenon of inactivation. It should be noted that this physical picture was mainly inferred from the mathematical descriptions of ionic conductance. Indeed, Hodgkin and Huxley cautiously note that "the physical basis for the equations should be only used for illustrative purposes and is unlikely to be the correct picture of the membrane." Nonetheless, these concepts revolutionized our way of thinking about electrical properties of membranes and laid the foundation for future mechanistic studies.

Macroscopic current measurements cannot uniquely discriminate between gating models with different rate constants (e.g., 1:1:1 or 1:2:3), as the predicted Na^+ currents would be virtually indistinguishable from the original (Armstrong, 1981). Thus, to constrain models of Na_v channel gating, it is necessary to monitor time and voltage-dependent distributions of nonconducting channel states. Therefore, the discovery of "gating currents" in the early 1970s made it possible to probe gating transitions even when the channel is closed or inactivated (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1973, 1974; Meves, 1974). "Gating current" refers to the transient current generated by the movement of voltage-sensing charges or dipoles within the electric field. The activating ON (outward) gating currents of Na_v channels in squid axon show two components, with the fast component being clearly related to channel opening, and the second, slower ON gating component was observed to be faster than inactivation. This led Armstrong and Bezanilla to propose that inactivation is not directly caused by the movement of a voltage-sensing inactivation particle as was proposed by the HH model (Armstrong et al., 1973; Armstrong and Bezanilla, 1974, 1977; Bezanilla and Armstrong, 1977).

The HH model also predicts that the OFF gating current will be unaffected by the state of inactivation, but it was observed that inactivation results in "immobilization" of roughly two thirds of the total OFF gating currents

(Armstrong and Bezanilla, 1973). These findings support a foot-in-the-door–type mechanism for inactivation, a key tenet of the coupled inactivation model (Fig. 2). Accordingly, reclosure of the activation gate is hindered by an inactivation particle, which binds near the channel entrance thereby preventing the return of coupled voltage-sensing charges.

Single-channel recording techniques allowed ion channel biophysicists to extract information about the various microscopic rates during gating transitions. Aldrich, Corey, and Stevens found that single Na_v channels from neuroblastoma cells primarily open once during a depolarizing voltage step with a mean open time that is not voltage dependent (Aldrich and Stevens, 1983, 1987; Aldrich et al., 1983). This indicates that entry into absorbing inactivated states is both rapid and voltage independent, as predicted by Armstrong and Bezanilla. However, by measuring the first latency to channel opening, they also discovered that a large fraction of Na_v channels open after the macroscopic current reaches its peak. These studies highlighted the fact that the macroscopic activation and inactivation kinetics are not solely a measure of microscopic channel opening and inactivation rates.

Other studies, including those by Vandenberg and Bezanilla (1991a,b), suggested that the final transition that leads to channel opening is slower than predicted by earlier models, but the microscopic rate constants for inactivation were still slower than activation rate constants. In a landmark single-channel study of Na_v channels, Vandenberg and Horn (1984) introduced the idea of using statistical methods such as maximum likelihood analysis to rigorously discriminate between different kinetic models by direct fitting single-channel records. Their analysis showed that a simple model of Na_v channel gating requires both open- and closed-state inactivation (see also Aldrich and Stevens, 1983). Furthermore, they found that wild-type channels have a long dwell time (2–5 ms) and open on multiple occasions. This is in contrast to the findings of Aldrich and Stevens (1987), who observed a short dwell time (0.2–1 ms) and only one channel opening before entering into the absorbing inactivated state. The seemingly opposing conclusions about inactivation being slow (Vandenberg and Horn, 1984) or fast (Aldrich and Stevens, 1983, 1987) may have a simple but intriguing explanation. Vandenberg and Horn (1984) performed their experiments using inside-out patches in which Na_v channel open times were severalfold longer when compared with cell-attached patches as used by Aldrich and Stevens (1983, 1987). Therefore, it seems that these groups may have been working on different states of the Na_v channel in which patch excision altered inactivation rates, a phenomenon that has yet to be fully explored. Although longer dwell times (1–2 ms) were also observed in a more recent study involving single-channel recordings of Na_v channels in inside-out patches (Goldschen-Ohm et al., 2013), multiple openings were not observed, suggesting that bursting behavior may not be a common feature for all Na_v channels. Studies of macroscopic Na^+ currents by Kuo and Bean (1994) showed that the channels are able to deactivate at least partially before recovering from inactivation. This idea is not incompatible with charge immobilization studies, where it was shown that approximately one third of the total charge remains free to move upon inactivation, and thus could account for rapid partial deactivation.

In Na_v channels, both activation and inactivation occur in an overlapping voltage range, which limits our ability to develop well-constrained gating models. Rapid entry into absorbing inactivated states masks the intrinsic lifetimes of open states, and limits the ability to unambiguously resolve the kinetics of slower or less frequent transitions in the activation pathway. One possible approach is to study activation gating in isolation by generating channels genetically deficient in inactivation (West et al., 1992; Wang et al., 2003). This experimental paradigm was implemented successfully to characterize the gating properties of the *Shaker* K_v channel and resulted in some of the most well-constrained gating models of voltage-gated ion channels to date (Zagotta et al., 1994).

Photoaffinity labeling using specific Na_v channel toxins (also see Pharmacology section below) identified a large molecular weight component (Beneski and Catterall, 1980), which led to the elucidation of the primary structure of Na_v channels (Noda et al., 1984). This major accomplishment set the groundwork for molecular and mutagenic studies that revolutionized the understanding of Na_v channels by assigning for the first time distinct functional gating roles to regions or residues. Ensuing cysteine accessibility studies on the skeletal muscle Na_v channel isoform $Na_v1.4$ showed that Cys residues in DIVS4 are rapidly modified by MTS reagents in a state-dependent manner, providing the first direct evidence that voltage-sensing charges translocate during the gating process (Yang and Horn, 1995; Yang et al., 1996).

Extensive mutagenic analysis of voltage-sensing charges of the Na_v channel failed to reveal a clear picture of the role of specific domains (Chahine et al., 1994; Yang et al., 1996; Kontis et al., 1997; Lerche et al., 1997; Kühn and Greeff, 1999). Mutations of charged residues in all the domains were found to affect activation, whereas those in S4 segments of primarily DI and IV had most effect on fast inactivation. Peptide toxins such as Anthopleurin-B were observed to dramatically reduce fast inactivation and suggested that an extracellular site may be linked to fast inactivation (Hanck and Sheets, 1995; Sheets and Hanck, 1995). Subsequent structure–function studies localized such toxin-binding sites to extracellular loops of DIV of the Na_v channel (Rogers et al., 1996; Benzinger et al., 1998).

Measurements of voltage-sensor kinetics by tagging them with fluorescent reporters showed that VSDIV moves fivefold slower than those in the first three domains (Chanda and Bezanilla, 2002). The time course of the activation of this voltage sensor is correlated with onset of inactivation and with the slow ON gating charge movement. However, single-channel studies in an inactivation-deficient mutant showed that DIV is not the inactivation particle itself, but its movement causes a secondary conformational change in the pore (Goldschen-Ohm et al., 2013). This slower opening presumably gives rise to the slow activation observed by Aldrich, Corey, and Stevens in their single-channel studies (Aldrich et al., 1983). Single-channel studies (Goldschen-Ohm et al., 2013) also showed that upon opening, Na_v channels have an ~75% chance of entering the subconductance state, suggesting that the channels preferentially undergo transition from open to a subconductance state. Thus, according to the asynchronous gating model, the activation of VSDI–III causes initial channel opening, whereas the subsequent activation of VSDIV uncovers a site for binding inactivation particle in the pore (Fig. 2). Inactivation follows rapidly once this site becomes available; therefore, the second opening is obscured in wild-type channels. Disabling DIV–S4 voltage sensing by introduced glutamine residues at the first three charge-carrying residues slows entry into, and recovery from, fast inactivated states (Capes et al., 2013). Collectively, these studies demonstrate that activation of VSDIV is both rate limiting and sufficient for Na_v channel inactivation.

Structure–function studies involving swaps of various Na_v channel VSD regions into a K_v channel background showed that DIV VSDs are intrinsically slower (Bosmans et al., 2008). By comparing the sequences of K_v and Na_v channels, Lacroix et al. (2013) were able to identify

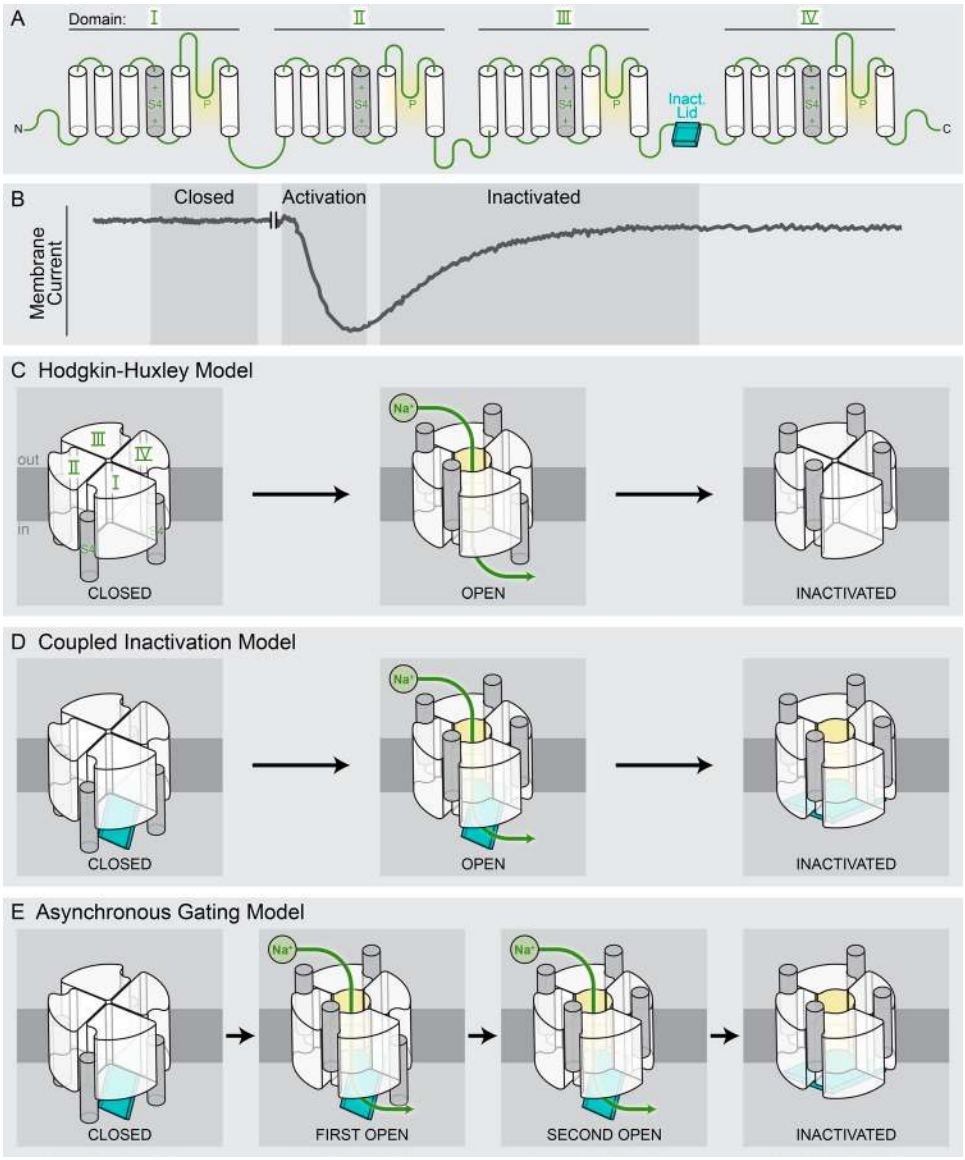

Figure 2. Schematic representation of gating models of eukaryotic sodium channels. (A) Transmembrane topology of a eukaryotic Na_v channel. The S4 voltage-sensing segment is shaded in gray, and the P-loop constitutes the selectivity filter region. The inactivation motif (cerulean-colored box) is the loop connecting domains III and IV. (B) Representative membrane currents through a voltage-activated sodium channel in response to a depolarizing pulse from a holding potential of −90 mV. The start of the depolarization pulse is represented as a break, and the gating current component has been subtracted. (C) Schematic rendering of the original HH model of sodium channel gating. Rapid activation of three "m" particles is sufficient for the channel to open, and slower activation of the "h" particle causes the channel to inactivate. (D) In the coupled inactivation model, activation of all four voltage sensors contributes to the channel opening. Inactivation results from binding of the inactivation lid to its receptor in the pore, which becomes accessible in the open state. (E) According to the asynchronous gating model, the activation of the first three VSDs of the sodium channel is sufficient to open the channel. Slow activation of the domain IV voltage sensor results in a secondary open state and makes the receptor for inactivation lid accessible.

speed control residues in S2 and S4 segments as the primary determinants for asynchronous activation of the voltage sensors of the Na_v channel. Despite significant progress in the past decade, many features of the asynchronous gating model remain unclear. For instance, we do not fully comprehend the structural dynamics involved in coupling activation of VSDIV to inactivation. Future studies combined with new structural information will undoubtedly shed more light on this asynchronous gating mode, which is likely to be a common feature in all pseudo-symmetric channels in the voltage-gated ion channel superfamily (Palovcak et al., 2014).

The $BacNa_v$ structures did confirm the VSDs to be hourglass-shaped four-helical bundles that contain intracellular and extracellular aqueous clefts lined by conserved acidic and polar residues (Fig. 3, A and B). The S4 helices are studded with conserved arginine-gating charges found in a characteristic RxxR motif (Payandeh et al., 2011; Zhang et al., 2012b), and a conserved hydrophobic constriction site forms a gasket around the gating charges as they transit through the "gating pore" (Fig. 3 B). The $BacNa_v$ VSD structures are consistent with classical models of Na_v channel function, where S4 arginine gating charges exchange ion-pair partners along the VSD during activation and deactivation, but some $BacNa_v$ channel gating charges also make compensating interactions to the protein backbone along the VSD (Fig. 3 B), suggesting that noncanonical gating charge interactions may also be functionally relevant.

Although the details of S4 motion in each VSD of eukaryotic Na_v channels remain unclear, it is expected that these movements are analogous to those in other voltage-sensing channels. The original models of voltage sensing (Armstrong, 1981) proposed that positive charge movement across the bilayer must be facilitated by negative charges in other parts of the protein or even negative lipid head groups (for a detailed discussion see Chowdhury, 2015). These concepts were further refined to suggest that the S4 helix undergoes a helical screw motion so that the charge pairing and α helicity are maintained during activation (Catterall, 1986; Guy and Seetharamulu, 1986; Yarov-Yarovoy et al., 2012). Although the recent structures of the voltage-sensing phosphatase suggest that this may be the case (Li et al., 2014), structures of other members of the voltage-gated ion channel superfamily suggest that the S4 helix may undergo a transition to a 3_{10} helix, in which case there is no necessity of a screw helical motion (Clayton et al., 2008). It is worth mentioning that despite a growing body of available data, there is no general consensus regarding the specific mechanism of charge movement, and it is unclear to what extent the details of this process are conserved throughout the voltage-gated ion channel family.

The $BacNa_v$ channel structures have also helped to define the molecular footprint of VSDs and pinpoint functionally unique residues within eukaryotic Na_v channel VSDs (Palovcak et al., 2014; Pless et al., 2014). Intriguingly, the Na_vAb and rat $K_v1.2$ channel VSDs share highly similar core structures (Payandeh et al., 2011), whereas the VSDs of Na_vRh display a remarkable "down shifting" of the S1–S3 regions around the S4 helix (Zhang and Yan, 2013; Payandeh and Minor, 2015). This observation suggests that the S1–S3 helices might not be totally constrained during activation. Moreover, a swinging motion of the VSDs within the plane of the membrane is also observed when the PMs of Na_vAb and Na_vRh (or $K_v1.2$) structures are superimposed (Fig. 3 C), highlighting potential transitions involved in channel activation or inactivation processes.

Pore gating. Pore gating in the voltage-gated ion channel family can occur either at the distal S6 hydrophobic bundle (del Camino et al., 2000; del Camino and Yellen, 2001) or in the selectivity filter region in CNG (Contreras et al., 2008) and BK channels (Chen and Aldrich, 2011; Zhou et al., 2011). Early studies with Na_v channels suggested that the quaternary strychnine can bind to its pore-blocking site from the cytoplasmic side only when channels are open, analogous to TEA block of K_v channels (Cahalan, 1978; Cahalan and Almers, 1979b). This was supported by discovery of the open pore blocker–like activity of the β4 subunit, which may also be a cytoplasmic blocker (Raman and Bean, 2001).

As anticipated from physiological studies on eukaryotic Na_v channels (Hille, 2001), the $BacNa_v$ channel PM contains a funnel-shaped extracellular vestibule, a narrowed selectivity filter, a large central cavity, and an intracellular activation gate (Fig. 3 A; Payandeh et al., 2011). Consistent with the view that an intracellular activation gate can regulate drug or blocker access (Hille, 2001), the structures of $BacNa_v$ channels are occluded to varying extents in this region (Fig. 3 D; Payandeh et al., 2011, 2012; McCusker et al., 2012; Zhang et al., 2012b; Shaya et al., 2014). Direct evidence for location of the pore gate in eukaryotic Na_v channels came from studies probing state-dependent accessibility of substituted cysteines in the S6 of DIV in an inactivation-deficient background (Oelstrom et al., 2014). Removing inactivation is essential to definitively establish that the observed accessibility changes are not caused by the inactivation particle blocking access to the substituted cysteines. It was shown that an evolutionary conserved patch of hydrophobic residues gate access to the sodium channel ion conduction pathway (Oelstrom et al., 2014). Strikingly, comparison of the structures of "closed" and "open" $BacNa_v$ channel PMs shows that a hydrophobic residue at an equivalent position is part of a narrow constriction (∼3.8 Å) in the access pathway and should form a steric barrier for hydrated ions. In addition to the conserved hydrophobic gate, the structures of other $BacNa_v$ channels suggest additional sites for putative

intracellular gates (Shaya et al., 2014). Accessibility studies in these bacterial channels will clarify whether these other gates are physiologically relevant. Furthermore, toxin-binding studies in eukaryotic Na_v channels suggest that there are likely to be additional conformational changes in the outer pore (Capes et al., 2012). However, it remains unclear to what extent these conformational changes in the outer pore contribute to the gating process. Interestingly, the first reported Na_vAb channel structure (Ile217Cys) revealed an essentially fourfold symmetric arrangement (Payandeh et al., 2011), whereas a subsequently determined Na_vAb channel structure (wild type) displayed an asymmetric collapse of the activation gate, central cavity, and selectivity filter, as well as a repositioning of the VSDs around the channel (Payandeh et al., 2012). These structural changes appear propagated through highly conserved residues forming a "communication wire" within the PM, and many analogous residue positions have been implicated in the slow inactivation process in eukaryotic Na_v channels (Payandeh et al., 2012). In this light, the $BacNa_v$ channels may provide a template to understand how the selectivity filter, central cavity, activation gate, and VSDs may be coupled in eukaryotic Na_v channels.

Electromechanical coupling. Our understanding of the molecular machinery involved in coupling VSD movements to the pore opening comes mostly from studies on homotetrameric K_v channels (see Blunck and Batulan, 2012; Chowdhury and Chanda, 2012). Mutations at multiple positions in the S4–S5 linker region and adjacent S6 helix have been shown to disrupt electromechanical coupling. In the Na_v channel, two different approaches were used to probe coupling between VSDIII and pore gates. By simultaneously monitoring the movements of the DIII voltage sensor and pore opening, Muroi et al. (2010) were able to identify multiple residues in this region as likely sites mediating electromechanical coupling. Further, by analyzing the derivatives of response curves, they were able to highlight several key residues as those that are involved in interactions in both resting and activated states.

Previously, it had been shown that lidocaine binding to the pore causes large hyperpolarizing shifts in VSDIII, making it harder to return to the resting state (Arcisio-Miranda et al., 2010), akin to charge immobilization observed upon TEA binding to the pore in K_v channels (Armstrong, 1969). This suggests that lidocaine, much like TEA, prevents closure of the pore gates. Arcisio-Miranda et al. (2010) exploited this phenomenon to

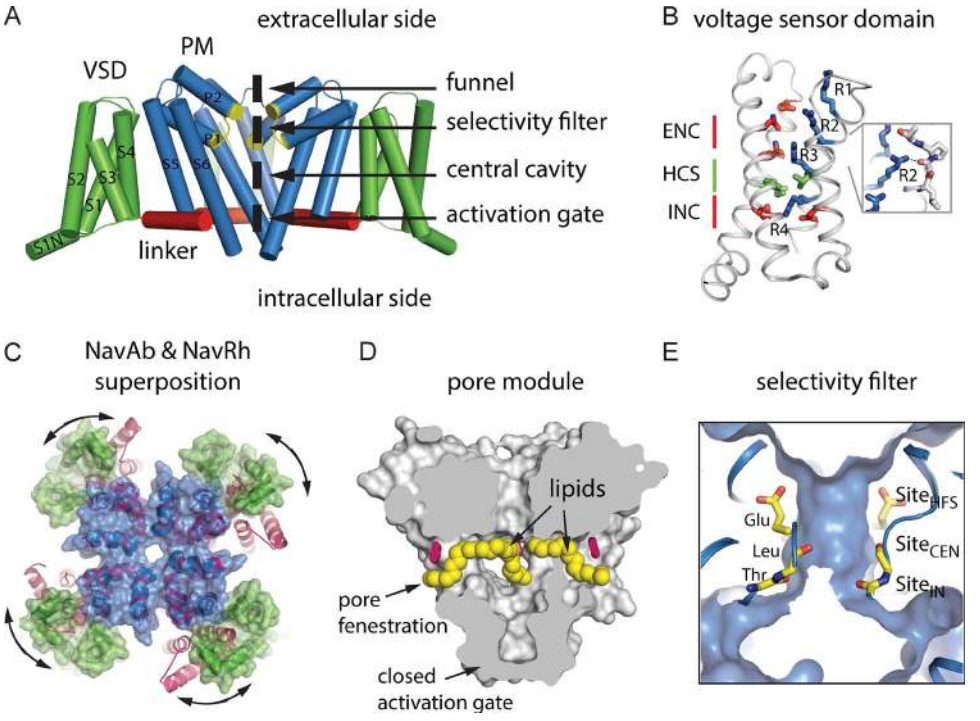

Figure 3. Overview of $BacNa_v$ crystal structures. (A) Side view of the Na_vAb channel (Protein Data Bank accession no. 3RVY) with the VSDs colored green, the S4–S5 linkers colored red, and the PM colored blue. The selectivity filter motif in all four subunits is colored yellow. Main regions within the pore structure are indicated, and the front VSD and pore domain are removed for clarity. (B) Voltage-sensor domain from Na_vAb highlights conserved structural and functional features within the VSD including the hydrophobic constriction site (HCS) and the intracellular and extracellular negative charge clusters (INC and ENC). The gating charges (arginine residues, R1–R4) are shown in blue sticks. (Inset) The R2 arginine gating charge hydrogen bonding with a backbone carbonyl from the S3 helix is highlighted. (C) Superposition of the Na_vAb and Na_vRh (Protein Data Bank accession no. 4DXW) channel pores (colored blue and pink, respectively) indicates the possibility of a significant movement of the VSDs within the plane of the membrane. (D) Side-view section of the Na_vAb channel shows locations of bound phospholipids (yellow spheres) within the PM and their penetration through the pore fenestrations. The side chain of Phe203 is shown in pink stick representation for reference, and the closed intracellular activation gate formed by the S6 helices is indicated. (E) Side view sectioned through the PM of Na_vAb shows three coordination sites identified within $BacNa_v$ selectivity filters. From the extracellular to intracellular side, these sites are: $Site_{HFS}$, $Site_{CEN}$, and $Site_{IN}$. The approximate positions of the Thr (T), Leu (L), and Glu (E) backbone or side-chain atoms from the conserved TLESW selectivity motif are also indicated.

examine if mutants in the S4–S5 linker and S6 region can allow VSDIII to return normally even when the pore is blocked by lidocaine, a possibility that could identify sites critical for maintaining coupling between the voltage sensor and pore. Strikingly, many of the identified high impact residues that disrupt the coupling between VSDIII and the lidocaine-binding site had been identified by Muroi et al. (2010).

Although these experimental paradigms have led to the identification of a subset of residues involved in electromechanical coupling, a deeper understanding of the fundamental mechanisms that determine coupling in these ion channels have remained elusive. The VSD and pore in Na_v and K_v channels are believed to be obligatorily coupled, which implies that standard allosteric analysis that would allow us to extract coupling energies is not applicable (Chowdhury and Chanda, 2012). This inability to estimate coupling free energies is a shortcoming that has to be overcome to obtain a quantitative understanding of how various structural features determine efficient voltage transduction from the VSD to the pore in these channels.

Fast inactivation gating. Perfusion of proteolytic enzymes in the squid axon preferentially removes inactivation while leaving activation intact, suggesting that the former likely involves proteinaceous components located on the cytoplasmic face of the channel (Armstrong et al., 1973). Furthermore, complementary Na_v channel fragments with a "clipped" linker between DIII and IV have impaired inactivation, implicating this loop in fast inactivation (Stühmer et al., 1989). This idea has been advanced by antibodies directed against residues 1491–1508 in the DIII–IV linker of neuronal Na_v channels, which antagonize inactivation of single channels (Vassilev et al., 1989). The implication of the DIII–IV linker has given rise to a working hypothesis that sodium channel inactivation proceeds through a "hinged-lid" mechanism, whereby linker residues serve as a molecular latch that interacts transiently with a receptor elsewhere in the channel (Fig. 2; Joseph et al., 1990). Consistent with this idea, mutation of the strictly conserved putative latch residues IFM to QQQ (also known as the Q^3 mutation) abolishes fast inactivation (West et al., 1992), whereas mutation of charged side chains or internal deletions are tolerated by inactivation (Moorman et al., 1990; Patton et al., 1992). In isolation, the ∼53–amino acid linker itself is largely disordered aside from a short α-helical structure found midway between the transmembrane tethers (Rohl et al., 1999; Sarhan et al., 2012), suggesting that it could be highly mobile. Although MTS-induced changes in gating or modification rates of introduced cysteine residues are consistent with local movement within the inactivation complex (Kellenberger et al., 1996; Lerche et al., 1997), investigations of conserved proline and glycine residues that might serves as "hinges" in DIII–IV to facilitate such movement are inconclusive (Kellenberger et al., 1997). The identity of the putative receptor for the IFM motif has proven to be elusive, but mutations in the pore-lining S6 segments of DI (Wang et al., 2003) and DIV (McPhee et al., 1994) can profoundly affect fast inactivation. However, such results need to be treated with caution, as mutations throughout the channel can also impact activation gating (Chahine et al., 1994; O'Leary et al., 1995; Chen et al., 1996; Smith and Goldin, 1997; Wagner et al., 1997; Jurkat-Rott et al., 2000, 2010; Keller et al., 2003; Motoike et al., 2004), which could indirectly alter inactivation because these processes are coupled.

Selectivity and permeation

Na_v channels drive excitability in the cardiovascular and nervous systems by rapidly gating the selective influx of Na^+. These moderately selective pores sit midway in their efficiency for namesake ion selectivity, allowing the mistaken passage of a wayward K^+ in 1 in 15 attempts as opposed to K_v channels, which mistake these two ions in roughly 1 per 100 attempts (Hille, 2001). This lower selectivity is possibly caused by the need only to depolarize the membrane, and therefore Na_v channels need not be as selective in the process. Unlike the clear multi-ion picture now available for K_v channels in which backbone carbonyls craft the selectivity filter (Doyle et al., 1998; Yellen, 2002; Long et al., 2005a), a comparable structure of the eukaryotic Na^+ selectivity filter and the chemical basis for this process remain unresolved. Yet, early experiments did reveal certain features that are consistent with a single Na^+ being bound to the channel most of the time (Hille, 1975a; Busath and Begenisich, 1982; Moczydlowski et al., 1984; Ravindran et al., 1992; French et al., 1994).

Substitutions within the putative selectivity filter have identified four key residues that are responsible for Na^+ selectivity, namely an aspartate (DI S5–S6 loop), glutamate (DII S5–S6 loop), lysine (DIII S5–S6 loop), and alanine (DIV S5–S6 loop; Favre et al., 1996; Sun et al., 1997; Huang et al., 2000). Within this DEKA motif, the presence of the positively charged Lys and the carboxylate from Glu seem to be vital components for maintaining an ionic permeability ratio of 0.03:0.075 for K^+ over Na^+. Based on an early molecular model of the Na_v channel pore, Lipkind and Fozzard (2008) ran molecular dynamics simulations and concluded that Na^+ selectivity hinges on both composition and conformation of the four non-identical selectivity filter residues. The underlying energetic mechanism may involve the interaction of Na^+ with glutamate (DII), thereby disrupting the interaction of its carboxylate with the amino group of the lysine in DIII and displacing it toward the alanine residue in DIV. To achieve this, Na^+ would only have to eliminate one or two waters from its hydration shell. Selectivity over K^+ would arise from the inability of this ion

to compete successfully with the lysine amino group (DIII), which would make an interaction with the glutamate in DII impossible.

Unlike eukaryotic Na_v channels, $BacNa_v$ channels lack the signature DEKA locus and hallmark binding of tetrodotoxin (TTX) but still retain Na^+ selectivity. However, it should be noted that key differences exist between selectivity mechanisms between eukaryotic and bacterial channels (Finol-Urdaneta et al., 2014). Nevertheless, in $BacNa_v$ channels, the S5 and S6 helices line the perimeter and central cavity of the PM, respectively, and are connected by a distinctive pore loop that forms a P1 helix–turn–P2 helix structure (Figs. 1 C and 3 A). This "turn" contains the $BacNa_v$ channel selectivity filter motif, which houses an extracellular acidic coordination site (TL**E**SWS in NavAb; $Site_{HFS}$) and two inner carbonyl coordination sites that line the central ion conduction pathway (**TL**ESWS in Na_vAb; $Site_{CEN}$ and $Site_{IN}$; Fig. 3 E). As predicted by permeation studies in eukaryotic Na_v channels (Hille, 1972), the selectivity filters in $BacNa_v$ channels is wide enough to accommodate Na^+ ions with their first hydration shell almost fully intact (Payandeh et al., 2011; McCusker et al., 2012; Tsai et al., 2013; Bagnéris et al., 2014; Shaya et al., 2014). Molecular dynamics simulations suggest that highly degenerate but favorable binding environments are able to concentrate two to three Na^+ ions within the selectivity filter and conduct them through a knock-on mechanism that favors hydrated Na^+ ions over hydrated K^+ or Ca^{2+} ions (Chakrabarti et al., 2013; Ulmschneider et al., 2013; Boiteux et al., 2014). Conformational isomerization of the acidic side chain within the selectivity motif (TL**E**SWS) has been further implicated in fostering an energetic landscape that promotes rapid diffusion of hydrated Na^+ (Chakrabarti et al., 2013; Boiteux et al., 2014; Ke et al., 2014), and analogous suggestions have been made about side chains within the DEKA selectivity locus of eukaryotic Na_v channels (Favre et al., 1996; Lipkind and Fozzard, 2000; Xia et al., 2013). It is worth noting that Na_vRh and Na_vAe channels have both captured an apparent hydrated Ca^{2+} within or above their respective selectivity filters, as these bound ions may represent physiologically relevant blocking sites (Zhang et al., 2012b; Shaya et al., 2014).

Three point mutations in the selectivity filter of Na_vAb that increase the amount of negatively charged residues produce a highly selective Ca^{2+} channel similar to those found in eukaryotic Ca_v channels (Tang et al., 2014). This observation is in concordance with results obtained from eukaryotic Ca_v channel mutagenesis, where it was shown that substitutions in the EEEE locus of the pore loop reduces ion selectivity by weakening ion-binding affinity. Unlike the degenerate binding mode of hydrated Na^+ ions proposed within the Na_vAb selectivity filter (TL**E**SWSM), crystal structures of the "Ca_vAb channel" variant revealed a linear arrangement of hydrated Ca^{2+} ions bound at two discrete high affinity sites by neighboring acidic side chains (TL**DD**WSD; Tang et al., 2014). This ionic arrangement would effectively screen away monovalent cations. Through a proposed knock-off mechanism, bound Ca^{2+} ions are released into the central cavity of Ca_vAb through a third low affinity carbonyl site (**T**LDDWSD) analogous to $Site_{IN}$ in Na_vAb (Fig. 3 E; Tang et al., 2014).

Unlike K_v channels, structural studies on $BacNa_v$ channels support the notion that both Na_v and Ca_v channels select and conduct hydrated cations. Interestingly, two highly conserved residues found in all Na_v and Ca_v channels (**T**LES**W** in Na_vAb) form an intersubunit hydrogen-bonding network in $BacNa_v$ channels that appears to hold the selectivity filter wide enough to accommodate hydrated cations (Payandeh et al., 2011). It has been suggested that these side-chain interactions (and therefore the structure of the selectivity filter) might be modulated by permeant ions, toxins, drugs, pathological mutations, and different gating states of the channel (Payandeh et al., 2012).

Pharmacology

Mechanisms of Na_v channel pharmacology will be discussed as well as the possible roles of membrane-facing fenestrations, long predicted, and now recently visualized in Na_v channel structures. We also propose a simplified nomenclature for Na_v channel toxin-binding sites and catalog the activities of key compounds.

Mechanisms of therapeutic inhibition by local anesthetics. Antiepileptic, antiarrhythmic, and local anesthetic compounds reduce Na_v channel activity with low and high affinity through "resting" and "use-dependent" inhibition mechanisms. In the clinical setting, this behavior is pharmacologically advantageous, as it allows for the systematic administration of Na_v channel inhibitors that primarily affect hyperexcitable tissues. Repeated stimulations produce conformational changes in the drug receptor that are concomitant with opening and channel inactivation that serve to further enhance drug interactions. Na_v channel drugs have been proposed to reduce conductance through a variety of overlapping mechanisms including pore block (Ramos and O'Leary, 2004), electrostatic interactions between the cationic charge on the drug and Na^+ at the selectivity filter (Barber et al., 1992; McNulty et al., 2007), and stabilization of fast or slow nonconducting states of the channel (Zilberter et al., 1991; Chen et al., 2000). In addition, local anesthetics cause gating charge immobilization (Hanck et al., 2000; Sheets and Hanck, 2003, 2005), primarily caused by long-range stabilization of VSDIII in the activated state (Muroi and Chanda, 2009).

In the simplest sense, use-dependence arises from enhanced interactions between the drug and open or inactivated channels, which in turn result in extended

residence times in inactivated and/or blocked states. As a result, molecules such as local anesthetics that are generally considered as blocking molecules can also act as gating modifiers. Although the basis for the resting or tonic blockade of the Na_v channel pore by drugs has been studied exhaustively, structures of the $BacNa_v$ channels may challenge some aspects of otherwise established mechanisms. Specifically, lateral openings within the PM of $BacNa_v$ channels create four large continuous access pathways, or fenestrations, that run perpendicular to the plane of the membrane and lead into the inner vestibule, the putative binding site for local anesthetics (Fig. 3 D; Payandeh et al., 2011, 2012). Molecular determinants analogous to the local anesthetic receptor site can be mapped onto solvent-exposed side chains within this large central cavity (Ragsdale et al., 1996; Pless et al., 2011), and bound drug-like molecules can also be localized nearby (Bagnéris et al., 2014). Remarkably, the lateral pore fenestrations are compatible with the passage of small neutral or hydrophobic drugs, and membrane lipid tails penetrate through these pore fenestrations in Na_vAb (Fig. 3 E; Payandeh et al., 2011). Although these pore fenestrations may change in size and shape during channel gating (Payandeh et al., 2012), how the $BacNa_v$ PM might compete with membrane lipids to gate and conduct Na^+ remains unanswered.

Nevertheless, the existence of such access pathways in Na_v channels was proposed in early work (Frazier et al., 1970; Strichartz, 1973), and this concept was conceptually streamlined by Hille (1977a,b), who proposed that local anesthetic drugs access a common central-binding site via distinct hydrophobic and hydrophilic pathways. One possibility is that once they traverse the membrane in the neutral form, the drugs act as a charged open channel pore blocker via a cytoplasmic pathway protected by the activation gate (Hille, 1977b). Alternatively, the neutral variant could also wedge its way into closed channels via hydrophobic access routes, which results in channel block after rapid protonation (Zamponi et al., 1993). However, neutral (e.g., benzocaine) or amphoteric blockers (e.g., lidocaine) rapidly inhibit channels when added to the extracellular solution, apparently, even while channels are closed (Hille, 1977b). To begin to differentiate between ultra-rapid intracellular blockade versus direct access via fenestrations, native single Na_v channels treated with pronase or batrachotoxin (BTX) to remove fast inactivation were studied and revealed very rapid blockade and a second discrete blocking event with much slower kinetics (Gingrich et al., 1993; Zamponi et al., 1993; Kimbrough and Gingrich, 2000). One intriguing possibility is that these distinct blocking events represent resting and use-dependent block, respectively. Notably, both rapid and discrete blocking events display strongly voltage-dependent rates and affinities, suggesting that the blocker hovers at a common site ~70% across the field, placing it at the inner limit of the selectivity filter. Consistent with this possibility, data show that local anesthetic block is reduced by increasing extracellular Na^+ or Ca^{2+} that could electrostatically reduce affinity by positioning within the selectivity filter (Hille, 1977b; Cahalan and Almers, 1979a; Wang, 1988). In addition to protein fenestrations within transmembrane segments within the bilayer, alternative pathways in the vicinity of the selectivity filter and upper S6 segment that provide extracellular access to the inner vestibule have been proposed (Qu et al., 1995; Sunami et al., 1997, 2000, 2001; Lee et al., 2001; Tsang et al., 2005). Collectively, these observations suggest that mutations can create, or at least modulate, intrinsic auxiliary fenestrations.

Site-directed mutagenesis has defined key residues along the pore-lining S6 segments in DIS6 (Yarov-Yarovoy et al., 2002), DIIIS6 (Yarov-Yarovoy et al., 2001), and DIVS6 (Ragsdale et al., 1994). Of note, channel inhibition by local anesthetics can be abrogated by the mutation of two conserved aromatic residues in the pore-lining DIVS6 segment. The application of nonsense suppression for the site-directed incorporation of noncanonical amino acids in cardiac and skeletal muscle Na_v channels has demonstrated that cation–pi interactions exist between lidocaine and QX-314 at aromatic residue ^{1579}Phe (^{1760}Phe in $Na_v1.5$), but not ^{1586}Tyr (^{1767}Tyr in $Na_v1.5$; Ahern et al., 2008; Pless et al., 2011). These energetically significant electrostatic interactions occur between a diffuse cation (most local anesthetics have a protonated subpopulation at physiological pH) and the negative electrostatic potential of the quadrupole moment of an aromatic side chain. Given that such interactions are geometrically restricted to occur between the face of the aromatic, not the edge, the data suggest that the inner vestibule S6 segments undergo a conformational change upon repeated depolarization and/or inactivation that reorients this aromatic side chain toward the permeation pathway.

Toxins that target Na_v channels. Given their contribution to action potential initiation, Na_v channels are principal targets of molecules present in animal venoms and plants (Kalia et al., 2015). As such, the use of toxins has led to the discovery of a variety of historical receptor sites in different Na_v channel regions (Catterall, 1980; Martin-Eauclaire and Couraud, 1992; Terlau and Olivera, 2004; Honma and Shiomi, 2006; Hanck and Sheets, 2007). Overall, toxins that alter Na_v channel function can do so through two separate mechanisms (Swartz, 2007; Bosmans and Swartz, 2010). First, pore-blocking toxins bind to the outer vestibule of the ion conduction pore to inhibit Na^+ flux (Hille, 2001). Second, gating-modifier toxins interact with a region of the channel that changes conformation during gating to influence opening or inactivation (Koppenhöfer and Schmidt, 1968a,b; Cahalan, 1975). Although certain gating-modifier toxins

can interact with both the pore region and one or more VSDs (Quandt and Narahashi, 1982; Tejedor and Catterall, 1988), their subsequent effect on Na_v channel function can typically be correlated with their ability to stabilize a VSD in a particular state. Notably, auxiliary β subunits help shape toxin sensitivity of Na_v channels, an emerging concept that may explain tissue-dependent variations in Na_v channel pharmacology and find use in the detection of functional β-subunit expression in normal and pathological conditions (Gilchrist et al., 2013; Zhang et al., 2013a). As opposed to using the often bewildering multitude of classic receptor sites (sites 1–9; Catterall et al., 2005), we will refer to the Na_v channel interaction site of animal toxins as either the pore region or the VSD, and we will further refine Na_v channel pharmacology based on the primary functional effects of toxins on channel function (Fig. 4).

Toxins influencing Na_v channel function by interacting with the pore region

TTX and saxitoxin (STX). TTX and STX are naturally occurring guanidinium toxins that potently interact with the Na_v channel pore region and cork the Na^+ permeation pathway (Furukawa et al., 1959; Narahashi et al., 1964; Moore et al., 1967; Narahashi, 1974; Hille, 1975b, 2001). TTX played an important role in the biochemical purification of the Na_v channel protein (Agnew et al., 1978; Miller et al., 1983) and in characterizing its selectivity filter (Terlau et al., 1991; Lipkind and Fozzard, 2008). Moreover, structural information about TTX and STX was used to predict the diameter of the Na_v channel pore, thereby providing powerful insights into the molecular organization of this ion channel family that still hold up today (Woodward, 1964; Hille, 1975b; Schantz et al., 1975; Payandeh et al., 2011, 2012; McCusker et al., 2012; Zhang et al., 2012b). Recently, STX returned to the spotlight when fluorescent derivatives were synthesized (Ondrus et al., 2012). These reagents enable real-time imaging of Na_v channels in live cells at the single-molecule level. Currently, TTX is used to divide the Na_v channel family into two groups based on their sensitivity toward the toxin; TTX-sensitive channel isoforms ($Na_v1.1$–$Na_v1.4$, $Na_v1.6$–$Na_v1.7$) are inhibited by nanomolar concentrations, whereas $Na_v1.8$ and $Na_v1.9$ require millimolar amounts to be blocked completely (Catterall et al., 2005). Although $Na_v1.5$ inhibition requires intermediate micromolar concentrations, TTX sensitivity can be substantially increased by replacing a cysteine in the domain I S5–S6 loop with a hydrophobic or aromatic residue (Lipkind and Fozzard, 1994; Leffler et al., 2005). Although this region of the selectivity filter plays a role in STX binding as well, other important extracellular residues have been implicated in forming the STX receptor site, most likely because of supplementary interactions with the second guanidinium group within the toxin (Fozzard and Lipkind, 2010).

BTX and veratridine (VTD). The steroidal alkaloid BTX is found in the excretions of poison dart frogs and certain bird species (Tokuyama et al., 1969; Dumbacher et al., 2000, 2004). BTX irreversibly inhibits fast and slow inactivation and shifts the voltage dependence of activation to more negative potentials, resulting in persistent Na_v channel activation. In addition, toxin-modified channels have a reduced single-channel conductance and an altered ion selectivity pattern, perhaps caused by a widened selectivity filter. The receptor site for BTX involves residues in multiple S6 pore segments and partially overlaps with that of local anesthetics (Linford et al., 1998; Wang and Wang, 1998; Wang et al., 2000; Du et al., 2011). Unlike lidocaine, which inhibits Na^+ currents, BTX is thought to partially occlude the ion permeation pathway, thereby leaving enough room for a fraction of Na^+ to get through (Catterall, 1975; Huang et al., 1982; Quandt and Narahashi, 1982; Wasserstrom et al., 1993; Linford et al., 1998; Wang and Wang, 1998; Bosmans et al., 2004). Because of its high affinity, radioactive BTX has been used extensively for Na_v channel identification in tissues and vesicles, and in screening potential therapeutics (Cooper et al., 1987; Gill et al., 2003).

The alkaloid toxin VTD is found in *Liliaceae* plants and causes persistent opening of Na_v channels while reducing single-channel conductance (Ulbricht, 1998). Evidence that the VTD and local anesthetics receptor overlap comes from mutagenesis studies within the pore-forming S6 segments, which also demonstrate that local anesthetics-occupied Na_v channels do not bind VTD (Ulbricht, 1998). Because of its ability to open Na_v channels, VTD is used in drug-screening essays in which controlling the membrane voltage is impractical (Felix et al., 2004).

Brevetoxins and ciguatoxins. Although the molecular architecture of cyclic polyether compounds from dinoflagellates such as brevetoxins and ciguatoxins is remarkable, these compounds have been implicated in numerous seafood-related poisonings and massive fish and marine mammal fatalities (Lin et al., 1981; Murata et al., 1989; Lewis et al., 1991). Both toxin families potentiate Na_v channel opening while altering Na^+ permeability, possibly through an interaction with the S6 segment in domain I and the S5 segment in domain IV (Bidard et al., 1984; Benoit et al., 1986; Lombet et al., 1987; Trainer et al., 1994). From a chemical point of view, these ladder-like polyether toxins may partition in the membrane to complement a structural motif within the Na_v channel (e.g. α helix) by means of a hydrogen bond network, which may lead to their biological activity (Ujihara et al., 2008).

Cone snail toxins. Cone snail venoms potentially comprise 100,000+ compounds that target an array of ion

channels and receptors (Terlau and Olivera, 2004; Franco et al., 2006; Lewis et al., 2012). In addition to their use as pharmacological tools, conotoxins are currently being tested in clinical trials as therapeutics for a range of disorders (Bende et al., 2014; Kalia et al., 2015). A subset of cone snail toxins, the μ-conotoxins, has been shown to compete with TTX to inhibit ion flow through Na_v channels by interacting with the pore region (Bulaj et al., 2005; Zhang et al., 2006; Leipold et al., 2011; Wilson et al., 2011). μ-Conotoxins have been used extensively as structure–function probes, yielding results that can now be reinterpreted as structural information, and models are being refined. For example, experiments with GIIIA and $Na_v1.4$ provided evidence of a clockwise domain arrangement around the pore, a fundamental feature of the tertiary channel structure (Dudley et al., 2000; Li et al., 2001). In addition, the net positive charge on these peptides indeed allows them to participate in long-range electrostatic interactions over realistic distances, which can contribute to binding and to the blocking of ion conduction (Hui et al., 2002, 2003; Korkosh et al., 2014). Notably, μ-conotoxin–induced Na_v channel block is by a strategically placed electrostatic barrier mechanism and differs from other channel inhibitors such as the charybdotoxin and guanidinium toxin family, which occlude the narrow part of the pore. Finally, μ-conotoxins helped lay the foundation for studying voltage-dependent channel gating mechanisms, thereby defining early constraints on the relationship between the pore and the VSDs and suggesting that the S4 segments move outward during channel activation (French et al., 1996).

Certain μ-conotoxins (e.g., KIIIA, GIIIA) do not occlude the pore completely, thereby leaving a residual current that can be blocked by TTX (Bulaj et al., 2005; Zhang et al., 2007, 2009, 2010; McArthur et al., 2011; Van Der Haegen et al., 2011). Detailed investigations on KIIIA revealed that this peptide can trap TTX in its binding site such that the guanidinium toxin cannot dissociate from the channel until the peptide does. Collectively, the possible interaction of guanidinium toxins and μ-conotoxins raises interesting pharmacological applications. For example, μ-conotoxin analogues may prevent TTX or STX binding while still allowing for a substantial residual current. As a result, these compounds could serve as an antidote in life-threatening situations involving guanidinium toxin poisoning (Zhang et al., 2009). Also, a sufficient diversity of conotoxins has been identified to assemble a pharmacological kit for distinguishing various Na_v channel isoforms in mammalian cells (Zhang et al., 2013b; Gilchrist et al., 2014). It is worth mentioning that auxiliary β subunits can

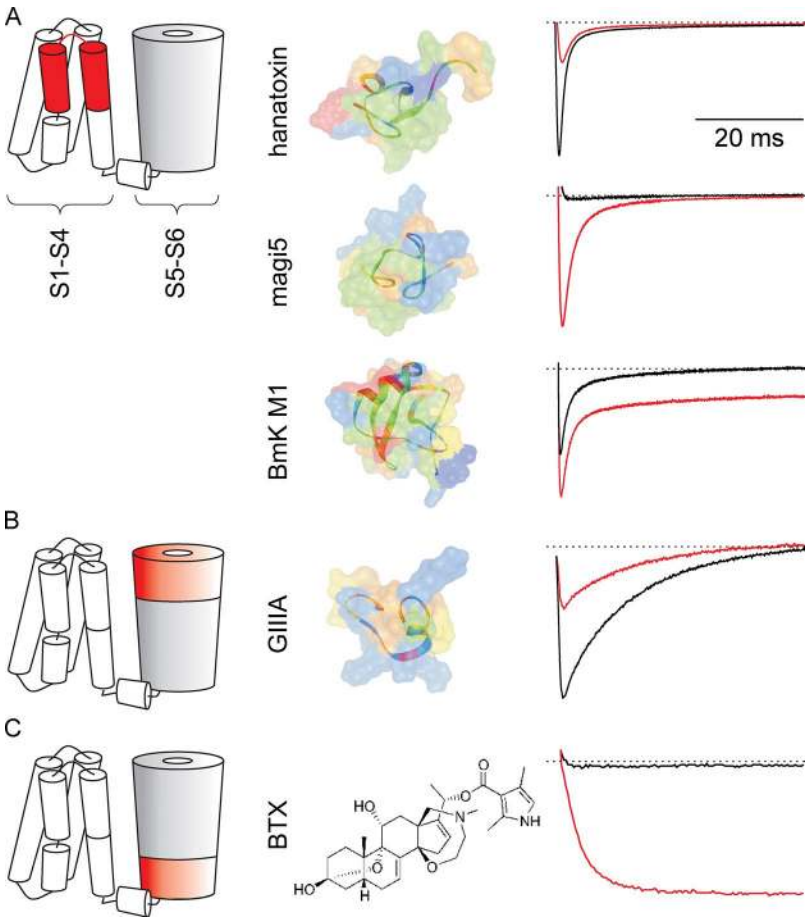

Figure 4. Interactions between animal toxins and Na_v channels. (A; left) Side view of a Na_v channel cartoon indicating the paddle motif (indicated in red) as the binding site for hanatoxin from the *Grammostola rosea* tarantula, Magi5 from the Hexathelidae spider *Macrothele gigas*, and BmK M1 from the *Buthus martensii* Karsch scorpion. (Middle) Structures of the three toxins colored according to residue class (green, hydrophobic; blue, positively charged; red, negatively charged; orange, polar). Toxin backbone is also shown. (Right) Effect of 100 nM hanatoxin (channel opening is inhibited), 1 μM Magi5 (channel opens at voltages where it is normally closed), and 100 nM BmK M1 (channel fast inactivation is inhibited) on $rNa_v1.2a$ channels expressed in *Xenopus laevis* oocytes and recorded with the two-electrode voltage-clamp technique. Despite binding to a similar region on the Na_v channel, each toxin has a different effect on channel opening or closing. Black trace represents control conditions, and red trace represents toxin. (B) Effect of 30 nM cone snail toxin GIIIA on $rNa_v1.4$-mediated currents recorded from *Xenopus laevis* oocytes. GIIIA blocks Na^+ flow by binding to the outer region of the pore mouth. (C) Effect of 1 μM BTX, isolated from the poison dart frog, on $rNa_v1.8$ channels expressed in *Xenopus laevis* oocytes. BTX binds to the inner region of the pore to drastically modify Na_v channel gating. Shown is the ability of BTX to open Na_v channels at voltages where they are normally closed and to inhibit fast inactivation. Black trace represents control conditions, and red trace represents toxin.

influence the kinetics of toxin block, thereby raising the possibility of using μ-conotoxins (or μO§-conotoxins such as GVIIJ) to detect the presence of β subunits in native tissues (Zhang et al., 2013a; Gajewiak et al., 2014; Wilson et al., 2015).

Toxins influencing Nav channel gating by interacting with the VSDs. In general, gating-modifier toxins interact with the S3b–S4 helix-turn-helix motif or paddle motif within each of the four Na_v channel VSDs (Gilchrist et al., 2014). The pharmacological importance of this distinct region was first established in K_v channels where mutations in the S3b–S4 loop altered channel sensitivity to hanatoxin, a founding member of the K_v channel gating modifier toxin family (Li-Smerin and Swartz, 2000). Later, this S3b–S4 motif was also identified in each of the four Na_v channel VSDs, and transplanting these regions from insect or mammalian Na_v to K_v channels resulted in functional K_v channels that are sensitive to Na_v channel toxins (Bosmans et al., 2008, 2011; Bende et al., 2014; Klint et al., 2015) (Fig. 4).

Scorpion toxins. Classic studies on scorpion venom established the presence of toxins capable of modulating Na_v channel voltage sensitivity (Koppenhöfer and Schmidt, 1968a,b; Cahalan, 1975; Martin-Eauclaire and Couraud, 1992). Based on the resulting functional effects, Na_v channel scorpion toxins were divided into two classes (Couraud et al., 1982). First, the α-scorpion toxins interact with VSDIV in a resting state, thereby limiting its movement upon membrane depolarization, resulting in the inhibition of fast inactivation (Jover et al., 1978; Rogers et al., 1996; Benzinger et al., 1998; Bosmans et al., 2008; Gur et al., 2011). Although their functional effects indeed imply a primary interaction with the DIV voltage sensor S3b–S4 paddle, antibody and photoaffinity–labeling studies as well as mutagenesis experiments on $rNa_v1.2a$ suggest that α-scorpion toxins can also interact with residues in the DI S5–S6 loop and the DIV S1–S2 loop (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Wang et al., 2011). However, a study by Campos et al. (2004) using Ts3 from *Tityus serrulatus* in concert with individually fluorescently labeled voltage sensors demonstrated an inhibitory effect on VSDIV movement as well as an effect on the voltage-dependent gating of DI, suggesting the notion of an allosteric coupling between adjacent DI and DIV.

β-Scorpion toxins promote channel opening by shifting the voltage dependence of activation to more hyperpolarized potentials. β-Scorpion toxins interact primarily with the DII S3b–S4 region and retain it in an activated state (Marcotte et al., 1997; Cestèle et al., 1998, 2006; Campos et al., 2007; Bosmans et al., 2008; Leipold et al., 2012). As a result of toxin exposure, the response of the channel to a subsequent depolarization may be enhanced, thereby resulting in a shift of the voltage dependence of activation to more negative voltages. Recently, the optical surface plasmon resonance technique was used to show that the DII and DIV S3b–S4 motifs can be isolated from rat $Na_v1.2$ and immobilized on sensor chips while remaining susceptible to particular scorpion toxins (Martin-Eauclaire et al., 2015). Although this label-free surface plasmon resonance method may be a powerful tool to detect interactions between ligands and Na_v channel paddles without the need to heterologously express the full-length channel, one expected limitation that emerged is an inability to detect ligand interactions that require regions outside of the paddle region (Cestèle et al., 1998, 2006; Leipold et al., 2006; Karbat et al., 2010; Zhang et al., 2012a).

Spider toxins. The list of Na_v channel spider toxins with comparable functionally important surfaces is growing rapidly, mostly because of the application of novel and sensitive screening techniques (Terstappen et al., 2010; Vetter et al., 2011; Gui et al., 2014; Klint et al., 2015). Interestingly, structure–function studies on SGTx1 from the *Scodra griseipes* tarantula with K_v channels and Magi5 from the hexathelid spider *Macrothele gigas* with Na_v channels reveal the functional importance of a cluster of hydrophobic residues surrounded by charged residues (Lee et al., 2004; Corzo et al., 2007). As a result of this amphipathic character, spider toxins are thought to partition in the membrane to interact with their receptor site within Na_v channel and K_v channel voltage sensors (Milescu et al., 2007, 2009; Swartz, 2008; Mihailescu et al., 2014). Although more experiments are required to clarify the influence of membrane lipids on toxin sensitivity of Na_v channels, it is not unreasonable to assume that spider toxins with comparable structures do not necessarily have similar membrane-interacting properties that may help determine their potency or target specificity (Gupta et al., 2015).

Depending on which VSDs they target and how those sensors couple to the overall Na_v channel gating process, spider toxins can have three distinct effects on Na_v channel function (Bosmans and Swartz, 2010). The first is for the toxin to inhibit channel opening in response to membrane depolarization (Middleton et al., 2002; Smith et al., 2007; Bosmans et al., 2008; Edgerton et al., 2008; Sokolov et al., 2008). A second outcome is for the toxin to hinder fast inactivation (Wang et al., 2008). Finally, the toxin can facilitate opening of the channel by shifting Na_v channel activation to hyperpolarized voltages (Corzo et al., 2007). After transferring S3b–S4 motifs within each of the four Na_v channel voltage sensors into K_v channels to individually examine their interactions with toxins (Bosmans et al., 2008), it became clear that the paddle motif in each of the four Na_v channel voltage sensors can interact with spider toxins, and that multiple paddle motifs are often targeted by a single toxin.

Sea anemone toxins. Mutagenesis studies have shown that cationic residues within sea anemone toxins are responsible for affinity differences between various Na_v channel isoforms (Barhanin et al., 1981; Gooley et al., 1984; Gallagher and Blumenthal, 1994; Khera and Blumenthal, 1996; Benzinger et al., 1998; Seibert et al., 2003; Norton, 2009). Because of their tight interaction with the S3b–S4 motif in VSDIV, sea anemone toxins potently inhibit Na_v channel fast inactivation (Romey et al., 1976; Catterall and Beress, 1978; Alsen et al., 1981; Rogers et al., 1996; Smith and Blumenthal, 2007). Typically, these toxins enhance recovery from inactivation without affecting Na_v channel activation, deactivation, or closed-state inactivation (Hanck and Sheets, 2007). Interestingly, sea anemone toxins bind with the highest affinity to the closed state of Na_v channels. This is surprising, as these peptides are generally hydrophilic in nature, and their binding site on the domain IV voltage sensor may be buried in the lipid membrane when the channel is closed. To reach their target, sea anemone toxins would therefore have to partition into the membrane, a feature observed with several spider toxins (Smith et al., 2005; Bosmans and Swartz, 2010).

Cone snail toxins. Cone snail venom contains toxins that alter Na_v channel gating by interacting with their voltage sensors. For example, μO-conotoxins are hydrophobic peptides that inhibit opening of $Na_v1.4$ and $Na_v1.8$ by preventing the activation of VSDII (Fainzilber et al., 1995; McIntosh et al., 1995; Daly et al., 2004; Leipold et al., 2007). Like certain β-scorpion toxins, the domain III pore loop may also play a role in μO-conotoxin interaction with $Na_v1.4$ (Zorn et al., 2006). Given their preference for the nociceptive channel $Na_v1.8$, this family of cone snail toxins is being tested in pain essays with the hope of finding novel analgesics (Ekberg et al., 2006; Gilchrist and Bosmans, 2012; Knapp et al., 2012; Teichert et al., 2012). δ-Conotoxins are structurally homologous to the μO-conotoxins but do not inhibit Na_v channel opening (Terlau et al., 1996). Instead, they inhibit Na_v channel fast inactivation, resulting in a prolongation of the action potential. Their mode of action suggests that δ-conotoxins can slow activation of VSDIV (Leipold et al., 2005). Finally, I-conotoxins shift Na_v channel activation to more hyperpolarized potentials, thereby causing these channels to open at voltages where they are normally closed (Buczek et al., 2008). These peptides differ from the μ-conotoxins and the δ-conotoxins in their action mechanism, the gene superfamily to which they belong, and the presence of unusual posttranslational modifications (Jimenez et al., 2003; Buczek et al., 2005; Fiedler et al., 2008).

Wasp toxins. Pompilidotoxins are small peptides purified from the venom of wasps that slow fast inactivation of neuronal Na_v channels but not $Na_v1.4$ and $Na_v1.5$ (Konno et al., 1998; Sahara et al., 2000). Site-directed mutagenesis of $Na_v1.2$ has revealed an important role for a glutamate residue in the DIV paddle motif in forming the toxin receptor site (Kinoshita et al., 2001). In concert, cationic residues within the pompilidotoxins were found to be critical for toxin activity (Konno et al., 2000). Because their chemical synthesis is relatively straightforward, these toxins are valuable tools to study Na_v channel gating.

Conclusion and future prospects

Na_v channels have played the role of biophysical muse for generations of membrane biophysicists. This has in turn driven fundamental advances on both experimental and theoretical fronts, and the future remains bright as new chemical and theoretical approaches are applied to every aspect of Na_v channel biology and pharmacology. The diversity of natural toxins that affect Na_v channel function will help elucidate the basics of channel gating while their therapeutic promise continues to develop. Moreover, the discovery of small-molecule compounds that bind to voltage sensors also represents an important development for isoform-specific therapeutics (McCormack et al., 2013; Ahuja et al., 2015). The development of chemical probes that report on activation and inactivation gating will produce new insights into function and will allow for a comparison of bacterial and eukaryotic Na_v channels. Furthermore, as these membrane proteins enter the new cryo–electron microscopy structural era, there is now the real possibility that Na_v channel aficionados will have high resolution structural data on eukaryotic Na_v channels to spark new predictions and validate old ones, as well as to inspire a new generation of excitable investigators.

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